Macrophage metabolic adaptation to heme detoxification involves CO-dependent activation of the pentose phosphate pathway

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Key Points

- Heme clearance in macrophages requires a metabolic shift to the pentose phosphate pathway (PPP).
- CO released by heme oxygenase (HMOX)-1 enzymatic activity is sufficient to induce the PPP *in vitro* and *in vivo*.
- Metabolic adaptation involving the induction of the PPP occurs in heme-clearing cells in a mouse model of sickle cell disease.

Abstract

Heme is an essential cofactor for numerous cellular functions, but release of free heme during hemolysis results in oxidative tissue damage, vascular dysfunction, and inflammation. Macrophages play a key protective role in heme clearance; however, the mechanisms that regulate metabolic adaptations that are required for effective heme degradation remain unclear. Here we demonstrate that heme loading drives a unique bioenergetic switch in macrophages, which involves a metabolic shift from oxidative phosphorylation toward glucose consumption. Metabolomic and transcriptional analysis of heme-loaded macrophages revealed that glucose is funneled into the pentose phosphate pathway (PPP), which is indispensable for efficient heme detoxification and required to maintain redox homeostasis. We demonstrate that the metabolic shift to the PPP is controlled by heme oxygenase-dependent generation of carbon monoxide. Finally, we show that metabolic adaptation is an essential component of macrophage-dependent heme clearance in a mouse model of sickle cell disease. Together, our findings demonstrate that metabolic adaptation to heme detoxification in macrophages requires a shift to the PPP that is induced by heme-derived carbon monoxide, suggesting pharmacological targeting of macrophage metabolism as a novel therapeutic strategy to improve heme clearance in patients with hemolytic disorders.

Keywords

Macrophage, metabolism, heme clearance, pentose phosphate pathway, carbon monoxide, sickle cell disease

Introduction

Hemolysis is prevalent in infections, genetic disorders such as sickle cell disease (SCD)[1] and glucose-6-phosphate dehydrogenase (G6PD) deficiency[2], and autoimmune-, trauma- or drug-induced hemolytic anemia[3]. Release of free heme into the circulation[4] damages the endothelium[5], promotes vascular inflammation[6], and exacerbates SCD[7,8], sepsis[9], and malaria[10,11]. Oxidative damage is a key driver of heme-induced pathology[12], and highly conserved antioxidant programs, including the upregulation of heme oxygenase 1 (HMOX1)[13,14] are at the core of the cellular response to increased free heme[15,16]. HMOX1 catalyzes the degradation of heme into biliverdin, iron, and carbon monoxide (CO). Activation of nuclear factor (erythroid-like 2)-like 2 (NRF2)[16], PPARγ[17], or CREB–AP-1 signaling[18,19], or inhibition of the repressor BACH1[20] regulate HMOX1 expression, and defects in antioxidant-response pathways result in heme-driven pathologies[21]. Moreover, HMOX1-deficiency in mice and humans reduces the capacity to withstand oxidative stress, and leads to the development of iron deficiency anemia, iron accumulation[22], chronic inflammation[23], and atherosclerosis[24,25].

Macrophages are specialized in heme clearance, controlling both the physiologic turnover of heme from senescent erythrocytes[26] and the response to pathological increases in free heme through uptake of heme-hemopexin[27], hemoglobin-haptoglobin[28], or heme itself[29,30]. Furthermore, macrophages are mediators of central iron metabolism[31,32] as well as iron homeostasis in peripheral tissues[33,34]. Metabolic adaptation is inextricably linked to macrophage function[35–37], however, the metabolic adaption necessary for heme detoxification in macrophages remains unexplored. Furthermore, it is not known whether heme itself, or catalytic products of heme degradation induce metabolic adaptation.

Here we examine the impact of heme loading on macrophage bioenergetics and investigate the mechanisms that regulate the cellular metabolic adaptations to promote heme clearance and detoxification. Our findings uncover a unique bioenergetic profile of heme-loaded macrophages and demonstrate a requirement for NADPH production, which is induced via a CO-dependent feed forward induction of the pentose phosphate pathway (PPP). This mechanism highlights the requirement for cellular metabolic

reprogramming in macrophages that acts in parallel with the canonical heme oxygenase pathway to promote heme degradation, redox homeostasis, and cell survival.

Methods

Complete experimental methods are included in the supplementary material.

Mice

Male C57BL/6J, B6.129X1-*Nfe2l2*^{tm1Ywk}/J (Nrf2 deficient), FVB.129S4(B6)-*Hif1a*^{tm1Jhu}/CkaMmjax (Hif1-α deficient), and *B6;129-Hbb*$^{tm2(HBG1,HBB*)Tow}$/*Hbb*$^{tm3(HBG1,HBB)Tow}$*Hba*$^{tm1(HBA)Tow}$/*J* (Townes Sickle Cell Disease) mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed at the Pinn Vivarium, University of Virginia, according to standard animal care practices dictated by the University of Virginia's Animal Care and Use Committee (ACUC). Animals had access to food and water *ad libitum* and were housed in a 12-hour light/dark cycle.

Bone Marrow Isolation and Culture

Bone marrow-derived macrophages (BMDMs) were cultured as previously described[38]. Briefly, bone marrow from the hind legs of 7-11-week old mice was isolated and incubated with 0.83% ammonium chloride to lyse erythrocytes. The remaining marrow was cultured in RPMI 1640 (Genesee Scientific, El Cajon, CA) containing 10% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA), 2% HEPES, 2% Antibiotic-Antimycotic (both from Gibco, Gaithersburg, MD), and 10% L929-conditioned media as a source of macrophage colony stimulating factor (L929 cells from American Type Culture Collection, Manassas, VA). Media was exchanged every 3 days, and on day 7 the media was exchanged for one lacking L929-conditioned media. BMDMs were then detached using 0.25% trypsin (Gibco), centrifuged, had their media refreshed, prior to analysis.

RAW Macrophage Culture

RAW264.7 cells were obtained from ATCC and cultured on Petri dishes in DMEM media (Genesee Scientific), with 10% fetal bovine serum (Atlanta Biologicals), 2% HEPES (Gibco), and 1% Antibiotic-Antimycotic (Gibco). Media was replaced every 3 to 4 days, and cells were allowed to grow to 90% confluency prior to splitting and passaging. Cells were separated from Petri dishes using 0.25% trypsin (Gibco).

Liver and Spleen Isolation

3 to 4 month-old Townes SCD mice were euthanized by carbon dioxide and blood was drawn via cardiac puncture. Blood was allowed to clot and serum was prepared by centrifugation for 5 minutes at 800 x *g*. Liver and spleens from these mice were then surgically excised. For assays involving protein, sections of approximately 50mg were homogenized in 1x RIPA lysis buffer (containing complete mini protease inhibitor) in a tissue homogenizer (Qiagen) for 10 minutes, before samples were centrifuged at 13000rpm for 5 minutes. The protein concentration of the supernatant was determined via Pierce BCA Assay (Thermo Scientific, Waltham, MA) prior to G6PD activity measurement.

mRNA Expression by RT-qPCR

RNA was isolated from BMDMs, tissue samples, or FACS-isolated cells with the RNEasy Mini Kit (Qiagen, Hilden, Germany), and libraries of cDNA were generated using the iScript cDNA Synthesis Kit (Biorad). Quantitative real-time PCR (RT-qPCR) was performed using Bioline SensiMix SYBR and Fluorescein Kit (Meridian Life Sciences, Memphis, TN) on a CFX Connect Real Time PCR System (Biorad). Primer pairs used were described previously or designed with PrimerBLAST and verified with melt-curve analysis. Sequences and references for primers used in this study can be found in Supplementary Table 1.

Metabolomics

Metabolomics analyses were conducted as described previously [38]. Briefly, BMDMs were treated with either vehicle or heme (10μM) for 6 hours. Cells were detached and centrifuged at 600 x *g* for 5 minutes. The supernatant was removed under vacuum, and the sample pellets stored at -80 °C before transport to Metabolon, Inc. (Durham, NC), who performed the global metabolomic analysis. Abundance data for all metabolites was normalized to sample protein content (as measured by Bradford Assay) and data are represented as a relative normalized abundance. Extended metabolomics methods can be found in the Supplementary Material.

Extracellular Flux Analysis

Extracellular flux analysis on BMDMs was conducted as previously described [38]. Briefly, BMDMs were seeded into a Seahorse 24-well culture plate (Agilent Technologies). Cells were allowed to adhere for 1-2 hours prior to treatment with vehicle or heme (10 or 50μM) for 6 hours. For assessing respiratory capacity, cells underwent a mitochondrial stress test (MST) as established previously[39]. For assessing the glycolytic capacity of the cells, a glycolytic stress test (GST) was performed to measure extracellular acidification rate (ECAR), representing the secretion of lactate into the extracellular media. Bioenergetics plots were created using the maximum respiratory capacity from MST (y-axis) and the glycolytic capacity from GST (x-axis) from parallel experiments in which the cells received identical treatments. Extended methods can be found in the Supplementary Material.

Protein Expression

Protein expression was quantified by Western blot. BMDMs were plated in 6-well plates and treated as indicated. Cells were lysed in ice-cold RIPA Buffer (Millipore, Burlington, MA) containing Complete Mini Protease inhibitor (Roche), sonicated for 30 seconds, centrifuged at 18000 x *g*, and the supernatant retained. Protein concentration was determined by Pierce BCA Assay (Thermo Scientific), and 50μg of each sample was digested in SDS PAGE loading buffer at 95°C for 5 minutes. Samples were run on a 10% SDS gel, transferred to nitrocellulose, stained with primary antibodies (see supplement), and imaged on a LI-COR Odyssey using fluorescently-tagged secondary antibodies (LI-COR Biosciences, Lincoln, NE).

Functional Cellular Assays

Functional assays were conducted on supernatants or whole cell lysates from BMDMs treated with vehicle, heme (10 or 50μM), CORM3 (50 or 100 μM) or iCORM3 (50 or 100 μM). Extended methods can be found in the Supplementary Material

Statistical Analysis

Data are represented as mean ± SEM, unless otherwise specified. Unpaired Welch's t-test determined statistical significance between two groups. For comparisons between more than two groups, One-Way Analysis of Variance tests were conducted to determine overall significance. Post-hoc Dunnett's Multiple Comparison test was used for experiments comparing multiple experimental groups to a single control, while post-hoc Tukey's tests were used for comparison between multiple experimental groups. For all experiments, α=0.05. Data analysis was performed using Prism 8 software (GraphPad Software, La Jolla, CA) and FCSExpress 6 (DeNovo Software, Pasadena, CA).

Results

Heme loading drives a bioenergetic shift in macrophages, reducing mitochondrial respiration and promoting glucose uptake

We established that exposure of murine bone marrow-derived macrophages (BMDMs) to free heme resulted in intracellular heme accumulation in a dose dependent manner (Figure 1A). Mitochondrial stress tests revealed a significant and dose-dependent suppression of oxygen consumption in "heme-loaded" macrophages (Figure 1B), with significant decreases in both basal and maximal mitochondrial respiration as well as reserve capacity. Heme loading induced a significant increase in uptake of the fluorescent glucose analog 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (Figure 1C) as well as a 2.5-fold increase in gene expression of the glucose transporter *Glut1* (Figure 1D). These data imply that heme-loaded macrophages use glucose as a fuel source. However, glycolytic stress tests revealed modest changes in glycolytic rate and capacity in heme-loaded macrophages (Figure 1E), and the bioenergetics phenogram illustrates that heme loading drives macrophages away from aerobic metabolism, with only a minor trend toward increased glucose utilization (Figure 1F). The dampened bioenergetic profile of heme-loaded macrophages resulted in a dose-dependent decrease in intracellular ATP levels (Figure 1G), despite demonstrating sustained cell survival (Figure 1H). Cell death was only observed at an extracellular heme concentration of 100μM, demonstrating a remarkable ability of BMDMs to withstand heme toxicity. Together, the significant increase in glucose uptake with minor changes in glycolytic rate imply that glucose is used in alternative metabolic pathways in heme-loaded macrophages.

Unbiased metabolomics analysis of heme-loaded macrophages reveals enrichment for metabolites associated with the pentose phosphate pathway

To investigate the changes in metabolic profile upon heme loading in macrophages, we used a mass spectrometry-based unbiased metabolomics approach (Metabolon, Inc., Durham, NC), which analyzed the relative abundance of over 500 cellular metabolites (Supplementary Table 2 provides a list of all significantly changed metabolites). Metabolomics analysis of BMDMs after 6 hours of heme loading

revealed enrichment for specific metabolic pathways, including heme degradation, dipeptide metabolism, the pentose phosphate pathway (PPP), and glycolysis (Figure 2A). Unsurprisingly, the most highly enriched pathway in heme-loaded macrophages was heme metabolism, with significant increases in heme and its degradation products biliverdin and bilirubin (Figure 2 A,B). Although heme loading increased glucose uptake (Figure 1C), initial metabolites such as glucose-6-phosphate, fructose-6-phosphate, and fructose-1,6-bisphosphate were not significantly increased, while several downstream glycolytic metabolites, including dihydroxyacetone phosphate, 3-phosphoglycerate, phosphoenolpyruvate, pyruvate, and lactate, were significantly enriched in heme-loaded macrophages (Figure 2C). These increases in glycolytic metabolites were not associated with changes in intermediates of the tricarboxylic acid (TCA) cycle after heme loading (Supplementary Figure 1A). However, metabolites associated with the oxidative reactions of the PPP, such as 6-phosphogluconate, ribulose/xylulose-5-phosphate, and ribose, were significantly more abundant in heme-loaded macrophages (Figure 2D). Isotopically labeled glucose studies in heme-loaded RAW macrophages also demonstrated a significant enrichment of $[^{13}C]_5$-ribose, as well as non-significant increases in metabolites at points of reentry from the PPP into glycolysis, without changes in any $[^{13}C]$ isotopologues of lactate (Supplementary Figure 1B), which paralleled the modest increase in glycolytic rate in heme-loaded macrophages (Figure 1E). Taken together, these results suggest that heme loading in macrophages redirects glucose metabolism to the PPP (Figure 2E).

Heme loading induces changes in metabolic gene expression to promote persistent pentose phosphate pathway flux and macrophage redox homeostasis

Immediate metabolic responses are mainly mediated by enzymatic activity, while prolonged changes in cellular metabolism are reflected in transcriptional activation of metabolic enzymes. To analyze changes in metabolic gene expression upon heme loading we exposed BMDMs for 6 or 24 hours with 1, 5, or 10μM heme. We found marked upregulation of heme degradation enzymes (*Hmox1*, *Blvra*, *Blvrb*) as well as genes involved in glucose uptake and phosphorylation (*Glut1*, *Hk1,* and *Hk2*) at both time points (Figure 3A and Supplementary Table 3). On the other hand, *Pfkp*, which encodes the phosphofructokinase

required to commit glucose-6-phosphate to glycolysis, was downregulated (Figure 3A). Concomitantly, enzymes that regulate the PPP, including glucose-6-phosphate dehydrogenase (*G6pd*) and 6-phosphogluconate dehydrogenase (*Pgd*), were upregulated after 6 hours (Figure 3B, upper panel), and remained significantly upregulated 24 hours after heme treatment (Figure 3B, lower panel). Furthermore, heme loading induced upregulation of genes responsible for shuttling PPP metabolites back into the glycolytic pathway (*Tkt*, *Taldo)* (Supplementary Figure 2A,B), while downstream glycolytic enzymes (*Gapdh*, *Pgk1*, *Pgam1*, *Pgam2*, *Pgam5*, *Eno1*, and *Eno2*), were upregulated only after 24 hours (Figure 3A). Together, these data demonstrate that heme loading results in a time-dependent, persistent metabolic shift that promotes glucose uptake, diverts carbon into the PPP, and returns the downstream metabolites to glycolysis (Figure 3C).

In response to external stimuli, macrophages adopt specific polarization states, which involves transcriptional upregulation of specific gene programs that allow them to respond appropriately[38,40,41] While heme loading produced modest upregulation of the M1 marker genes *Il6, Ptgs2, and Nos2* (Supplementary Figure 2C) as well as M2 markers, including *Il10, Il4,* and *Ym1* (Supplementary Figure 2D) , we observed a significant induction of marker genes for redox-responsive 'Mox' macrophages (Supplementary Figure 2E), which are typically induced by phospholipid oxidation products[42] and are known to have metabolic characteristics distinct from canonical M1 or M2 macrophage subtypes[38]. Consistent with this antioxidant response, we observed a marked increase in intracellular reactive oxygen species (ROS) in heme-loaded macrophages; however, this increase in ROS was unaffected by inhibition of G6PD with DHEA (Supplementary Figure 2F).

Expression of *G6pd* and *Pgd* was significantly and dose-dependently increased in heme-loaded wild type BMDMs (Figure 3D). This effect was independent of Nrf2, since heme loading induced the expression of *Glut1*, *G6pd*, and *Pgd* also in BMDMs isolated from *Nrf2*-deficient mice (Supplementary Figure 2G). In contrast, the response in *Hif1a*$^{-/-}$ BMDMs was lower compared with wild type (Supplementary Figure 2H), suggesting a role for HIF1α activity in the regulation of PPP gene expression upon heme loading.

G6PD and PGD use glucose-6-phosphate and its downstream metabolites to reduce NADP+ to NADPH[43], and heme loading increased the NADPH-producing activity of G6PD (Figure 3E). NADPH is required to reduce oxidized glutathione (GSSG), and pharmacologic inhibition of G6PD with dehydroepiandosterone (DHEA) significantly decreased NADPH levels (Figure 3F) and drastically increased intracellular GSSG (Figure 3G) in heme-loaded macrophages. Moreover, heme loading resulted in a significant decrease in the GSH/GSSG ratio, indicative of increased oxidative stress, which was significantly exacerbated upon inhibition of G6PD (Figure 3H). These data demonstrate that in heme-loaded macrophages, induction of the PPP is necessary to maintain cellular redox homeostasis.

G6PD activity is required for heme detoxification in macrophages

To examine whether the activity of the PPP was required for heme loading and/or degradation, we first loaded macrophages with 10μM heme for 1, 3, and 6 hours, which resulted in a time-dependent increase in intracellular heme accumulation for the first 3 hours, with a subsequent decrease, indicative of heme degradation. However, in the presence of the G6PD inhibitor DHEA, macrophages continued to accumulate heme, resulting in significantly higher intracellular heme levels at 6 hours (Figure 4A). These data indicate that G6PD activity controls the heme degradation phase, rather than impacting heme loading. Next, we preloaded macrophages with heme for 4 hours and monitored intracellular heme levels for an additional 12 hours. While vehicle-treated heme-loaded macrophages demonstrated steady decrease in intracellular heme levels, inhibition of G6PD by DHEA inhibited heme degradation (Figure 4B). Strikingly, G6PD inhibition decreased the heme degradation rate by 75% (Figure 4C). Moreover, pharmacologic inhibition of the PPP with 6-amininicotinamide (a PGD inhibitor) inhibited PPP gene expression induced by heme loading (Supplementary Figure 3A), as did inhibition of G6PD with DHEA (Supplementary Figure 3B). These data demonstrate that activity of the PPP is specifically required for heme degradation in macrophages (Figure 4D).

Carbon monoxide generated by HO-1-dependent heme breakdown triggers the metabolic switch to the pentose phosphate pathway

Pharmacological inhibition of HMOX1 activity blunted the increase in *Glut1* and PPP gene expression induced by heme loading (Supplementary Figure 4A), which indicated that a heme breakdown product may mediate changes in cellular metabolism. To test whether carbon monoxide (CO) can act as an inducer of the PPP in macrophages, we treated BMDMs with tricarbonylchloro(glycinato)ruthenium (II) (CORM3), a molecule that releases CO upon contact with water (Figure 5A). CORM3 significantly increased the expression of *Glut1*, *G6pd*, and *Pgd* compared to treatment with vehicle or previously inactivated CORM3 (iCORM) (Figure 5B), with a concomitant increase in NADPH producing activity (Figure 5C), and intracellular NADPH levels (Figure 5D). In contrast to heme loading, CORM treatment of BMDMs did not significantly induce either M1 (Figure 5E) or M2 (Figure 5F) marker gene expression. However, CORM treatment resulted in induction of expression of Mox markers *Gclm*, *Srxn1*, and *Txnrd1* (Figure 5G), suggesting that CO is inducing a distinct Mox macrophage phenotype. Since HMOX1 requires NADPH as a cofactor to detoxify heme, our data are consistent with the model in which CO derived from heme breakdown acts as a feed forward signal to promote a metabolic redirection essential for NADPH production (Figure 5H). Furthermore, CORM3 induced *Glut1* expression, which was significantly blunted in BMDMs from *Hif1a*-deficient mice (Supplementary Figure 4B). However, CORM3-induced expression of *G6pd* and *Pgd* was similar between *Hif1a*-deficient and wild type macrophages (Figure S4B), demonstrating that Hif1α is required for the CO-dependent upregulation of *Glut1*, while the upregulation of PPP enzymes by CO is independent of Hif1α.

Treatment of BMDMs with CORM3 significantly reduced basal and maximal respiration (Figure 6A), as well as extracellular acidification rate, indicating a decrease in glycolytic activity (Figure 6B). The suppression of both mitochondrial respiration and glycolysis by CORM3 drove macrophages to a bioenergetically suppressed state (Figure 6C), which may explain why heme loading does not induce the glycolytic phenotype characteristic of M1 macrophages [44]. As with heme loading, CORM3 treatment decreased intracellular ATP in a dose-dependent manner (Figure 6D). However, CORM3 treatment did not

downregulate expression of genes encoding components of the electron transport chain complexes (Supplementary Figure 5A), indicating that CO-induced decrease in oxygen consumption and ATP production were independent of changes in OXPHOS gene expression. Together these data demonstrate that CO mediates the metabolic changes characteristic of heme-loaded macrophages.

Macrophages undergo metabolic adaptation in clinically relevant models of heme clearance

During episodes of hemolysis, macrophages in the spleen and liver are responsible for detoxifying heme from the extracellular environment[29,45]. To examine the metabolic state of these organ systems in a model of chronic hemolysis, we used Townes Sickle Cell Disease transgenic mice (SCD mice), which express human β-globin instead of the endogenous mouse protein[46–48]. When compared with mice expressing normal human hemoglobin (β^A mice), mice expressing sickle hemoglobin (β^S mice) had significantly higher levels of free heme in the serum (Figure 7A). Compared with β^A mice, spleens of β^S mice showed increased mRNA expression of *Hmox1* and *Pgd*, and a trend toward increased *G6pd* expression (Figure 7B). Expression of *Hmox1*, *G6pd*, and *Pgd* was also significantly increased in the livers of β^S mice (Figure 7C), which corresponded to a significant increase in liver G6PD activity (Figure 7D). Remarkably, liver G6PD activity positively correlated with serum heme levels in β^S mice (Figure 7E). We then used flow-assisted cell sorting (FACS) to isolate cells expressing macrophage markers from spleens of β^A and β^S mice (representative gating strategy in Figure 7F). Interestingly, we observed that a larger proportion of the $CD45^+$ cells in the spleens of β^S mice were positive for macrophage markers including F4/80 and CD11b (Figure 7G). Furthermore, FACS-isolated macrophages demonstrated significantly higher expression of *G6pd* and a trend toward increased *Pgd* levels (Figure 7H). Since we observed that the PPP is upregulated locally in tissues where HMOX1 activity was increased, we tested the hypothesis that local production of CO is sufficient to upregulate the PPP. We treated wild-type mice with either saline or CORM3 (10mg/kg) for 7 days via intraperitoneal injection (Figure 7I), and examined expression of PPP genes in isolated peritoneal cells. Peritoneal cells from mice treated with CORM3 showed an increase in expression of *G6pd* and *Pgd* compared with cells isolated from mice that received saline control (Figure

7J). Taken together, these data demonstrate that increased free heme drives a metabolic adaptation that promotes the PPP and provides metabolic support for efficient heme clearance under conditions of chronic hemolysis.

Discussion

Efficient heme clearance is vital to prevent massive oxidative tissue damage in settings of hemolysis. Macrophages are specialized to deal with excessive free heme, and we demonstrate how heme-loaded macrophages adapt their metabolism in order to maintain redox homeostasis, to prevent cell death and to efficiently detoxify heme. While extracellular heme has been shown to act as a danger signal in pro-inflammatory contexts[7,49], the metabolic adaptations that allow macrophages to detoxify heme remained unclear. Here we show that intracellular free heme induces a specific metabolic shift in macrophages, which involves downregulation of oxidative phosphorylation and a minimal change in aerobic glycolysis despite increased uptake of glucose, which is utilized by the pentose phosphate pathway (PPP) at the apparent expense of decreased intracellular ATP. Upregulation of the PPP is controlled at the transcriptional level as well as by the function of metabolic enzymes, and pharmacologic inhibition of the PPP revealed that this metabolic pathway is essential for heme degradation in macrophages.

The PPP is a major source of the reducing equivalent NADPH[50], and we show that heme-loaded macrophages had increased activity of glucose-6-phosphate dehydrogenase, which resulted in increased NADPH production. Furthermore, pharmacologic inhibition of G6PD increased the level of accumulated heme, decreased the accumulation of NADPH, and prevented heme degradation by macrophages. As expected, heme increased oxidative stress within the cell via oxidation of glutathione. Additionally, inhibition of G6PD further increased the oxidation level in heme-loaded macrophages. Genetic deficiency of G6PD, the initial enzyme in the PPP and a key generator of cellular NADPH, is the most common inherited hemolytic anemia worldwide[2], and patients with G6PD deficiency are susceptible to hemolytic events after exposure to oxidative stresses such as infection or certain drugs[51]. Our data suggest that G6PD deficiency impairs the macrophage response to oxidative stress and underscore the importance of macrophage metabolic adaptation in maintaining homeostasis in response to free heme.

Macrophages are able to accumulate heme intracellularly at concentrations deleterious to other cell types and thus play an essential role in heme clearance[52]. Interestingly, blockade of NADPH production via inhibition of G6PD negated the heme-induced upregulation of the PPP, suggesting that the activity of this

pathway in response to heme results in positive feedback that stimulates its own upregulation. In response to heme, cells including macrophages upregulate the expression of *Hmox1*, which metabolizes heme to biliverdin in an NADPH-dependent manner, releasing Fe^{2+} and carbon monoxide (CO) in this process[53]. We show that HMOX1 activity is required for the upregulation of the PPP in heme-loaded macrophages, and that CO itself can induce the metabolic shift towards the PPP both *in vitro* and *in vivo*. In the absence of heme, CO drives macrophages toward a bioenergetically suppressed phenotype, similar to the profile we previously observed in Mox macrophages[38]. We further demonstrate that this "heme/CO-induced" metabolic switch to the PPP is independent of the activity of the transcription factors Nrf2 and Hif1α. CO has been shown to stimulate gene expression[54], and we show that addition of a CO-releasing molecule (CORM3) to BMDMs upregulates the expression of enzymes regulating the PPP (*G6pd*, and *Pgd)* and of *Glut1* in the absence of heme. Moreover, CO treatment produces changes in macrophage bioenergetics that parallel heme loading, and administration of exogenous CORM3 *in vivo* results in a local upregulation of the PPP. This is consistent with a model by which CO released via the activity of HMOX1 drives the shift in macrophage metabolism, suggesting a local feed-forward mechanism that is induced by heme catabolism itself. The mechanism(s) by which CO causes a metabolic switch to the PPP in macrophages remain unclear. CO is a highly active intracellular signaling molecule[55–58], and acts via several mechanisms, including direct binding to cytosolic enzymes, transcription factors such as Hif1α, and components of the electron transport chain. Previous studies in cancer cells have demonstrated that CO inhibits the H2S producing enzyme cystathionine beta synthase, which results in a loss of phosphofructokinase (PFK) FB3 stability. This results in lower intracellular levels of fructose-2,6-bisphosphate and a subsequent increase in glucose through the PPP[59]. Furthermore, CO has been shown to induce activation of HIF1α to promote a cytoprotective transcriptional program[60]; however, our results indicate that in heme-loaded cells, the induction of the pentose phosphate pathway is independent of HIF1α.

Finally, we show that the PPP is upregulated in macrophages in a mouse model of sickle cell disease (SCD). Cells of the reticuloendothelial system are responsible for the clearance of erythrocytes[61] and free heme[62]: red pulp macrophages in the spleen are responsible for removing damaged erythrocytes[63,64], while

monocyte-derived macrophages are recruited to the liver to support Kupffer cell-mediated clearance of erythrocytes and heme in SCD[65]. Our data show that the PPP is upregulated in the livers and spleens of SCD mice, and that the NADPH producing activity of the PPP is directly correlated with serum heme levels in these mice. We propose that metabolic adaptation is key for heme clearance by reticuloendothelial cells, and that metabolic adaptation of these cells is necessary to deal with the homeostatic stress of chronic hemolysis.

Together, our findings point to a central role of metabolic adaptation in cells that deal with excessive heme concentrations, and therefore, may be the basis for the identification of therapies that can be used to enhance efficient heme clearance in pathological settings such as hemolytic anemia, sickle cell disease, β-thalassemia, and in other settings where erythrocyte turnover is increased. Currently, CO therapy is being investigated as intervention in stroke[66], acute lung injury[67], ischemia-reperfusion injury[68,69], acute respiratory distress syndrome[70] and SCD[71,72]. Our findings point to an additional therapeutic potential of CO, with impact on heme clearance in SCD, sepsis[9], and malaria[10,11].

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Authorship

Contribution: N.L. conceived the project, designed experiments, analyzed data, and wrote the manuscript; G.F.P.B. designed and conducted experiments, analyzed data, and wrote the manuscript; S.Y. designed and conducted experiments, analyzed data, and wrote the manuscript; V.S. designed and conducted

experiments, analyzed data, and wrote the manuscript. T.E.F. conducted experiments and analyzed data. B.E.I. provided reagents and animal models, and analyzed data. All authors critically read, corrected, and approved the final version of the manuscript.

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References

1. Piel FB, Steinberg MH, Rees DC. Sickle Cell Disease. *N. Engl. J. Med.* 2017;376(16):1561–1573.
2. Cappellini M, Fiorelli G. Glucose-6-phosphate dehydrogenase deficiency. *Lancet*. 2008;371(9606):64–74.
3. Liebman HA, Weitz IC. Autoimmune Hemolytic Anemia. *Med. Clin. North Am.* 2017;101(2):351–359.
4. Ware RE, de Montalembert M, Tshilolo L, Abboud MR. Sickle Cell Disease. *Lancet*. 2017;390(10091):311–323.
5. Camus SM, De Moraes JA, Bonnin P, et al. Circulating cell membrane microparticles transfer heme to endothelial cells and trigger vasoocclusions in sickle cell disease. *Blood*. 2015;125(24):3805–14.
6. Almeida CB, Souza LEB, Leonardo FC, et al. Acute hemolytic vascular inflammatory processes are prevented by nitric oxide replacement or a single dose of hydroxyurea. *Blood*. 2015;126(6):711–20.
7. Belcher JD, Chen C, Nguyen J, et al. Heme triggers TLR4 signaling leading to endothelial cell activation and vaso-occlusion in murine sickle cell disease. *Blood*. 2014;123(3):377–90.
8. Kato GJ, Steinberg MH, Gladwin MT. Intravascular hemolysis and the pathophysiology of sickle cell disease. *J. Clin. Invest.* 2017;127(3):750–760.
9. Larsen R, Gozzelino R, Jeney V, et al. A Central Role for Free Heme in the Pathogenesis of Severe Sepsis. *Sci. Transl. Med.* 2010;2(51):51ra71-51ra71.
10. Dey S, Bindu S, Goyal M, et al. Impact of intravascular hemolysis in malaria on liver dysfunction: involvement of hepatic free heme overload, NF-κB activation, and neutrophil infiltration. *J. Biol. Chem.* 2012;287(32):26630–26646.
11. Ferreira A, Balla J, Jeney V, Balla G, Soares MP. A central role for free heme in the pathogenesis of severe malaria: the missing link? *J. Mol. Med.* 2008;86(10):1097–1111.
12. Deuel JW, Schaer CA, Boretti FS, et al. Hemoglobinuria-related acute kidney injury is driven by intrarenal oxidative reactions triggering a heme toxicity response. *Cell Death Dis.* 2016;7(1):e2064.

13. Lanceta L, Mattingly JM, Li C, Eaton JW. How Heme Oxygenase-1 Prevents Heme-Induced Cell Death. *PLoS ONE*. 2015;10(8):e0134144.
14. Wegiel B, Nemeth Z, Correa-Costa M, Bulmer AC, Otterbein LE. Heme oxygenase-1: a metabolic nike. *Antioxid. Redox Signal.* 2014;20(11):1709–1722.
15. Biswas C, Shah N, Muthu M, et al. Nuclear Heme Oxygenase-1 (HO-1) Modulates Subcellular Distribution and Activation of Nrf2, Impacting Metabolic and Anti-oxidant Defenses. *J. Biol. Chem.* 2014;289(39):26882–26894.
16. Ishii T, Itoh K, Takahashi S, et al. Transcription Factor Nrf2 Coordinately Regulates a Group of Oxidative Stress-inducible Genes in Macrophages. *J. Biol. Chem.* 2000;275(21):16023–16029.
17. Krönke G, Kadl A, Ikonomu E, et al. Expression of heme oxygenase-1 in human vascular cells is regulated by peroxisome proliferator-activated receptors. *Arterioscler. Thromb. Vasc. Biol.* 2007;27(6):1276–1282.
18. Krönke G, Bochkov VN, Huber J, et al. Oxidized phospholipids induce expression of human heme oxygenase-1 involving activation of cAMP-responsive element-binding protein. *J. Biol. Chem.* 2003;278(51):51006–14.
19. Wright MM, Kim J, Hock TD, et al. Human haem oxygenase-1 induction by nitro-linoleic acid is mediated by cAMP, AP-1 and E-box response element interactions. *Biochem. J.* 2009;422(2):353–361.
20. Igarashi K, Sun J. The heme-Bach1 pathway in the regulation of oxidative stress response and erythroid differentiation. *Antioxid. Redox Signal.* 2006;8(1–2):107–118.
21. Alam J, Cook JL. How Many Transcription Factors Does It Take to Turn On the Heme Oxygenase-1 Gene? *Am. J. Respir. Cell Mol. Biol.* 2007;36(2):166–174.
22. Kovtunovych G, Eckhaus MA, Ghosh MC, Ollivierre-Wilson H, Rouault TA. Dysfunction of the heme recycling system in heme oxygenase 1-deficient mice: effects on macrophage viability and tissue iron distribution. *Blood*. 2010;116(26):6054–62.

23. Kapturczak MH, Wasserfall C, Brusko T, et al. Heme Oxygenase-1 Modulates Early Inflammatory Responses: Evidence from the Heme Oxygenase-1-Deficient Mouse. *Am. J. Pathol.* 2004;165(3):1045–1053.
24. Ishikawa K, Sugawara D, Wang Xp A, et al. Heme oxygenase-1 inhibits atherosclerotic lesion formation in ldl-receptor knockout mice. *Circ. Res.* 2001;88(5):506–12.
25. Ishikawa K, Navab M, Lusis AJ. Vasculitis, Atherosclerosis, and Altered HDL Composition in Heme-Oxygenase-1-Knockout Mice. *Int. J. Hypertens.* 2012;2012:.
26. Alam MZ, Devalaraja S, Haldar M. The Heme Connection: Linking Erythrocytes and Macrophage Biology. *Front. Immunol.* 2017;8:.
27. Belcher JD, Chen C, Nguyen J, et al. Haptoglobin and hemopexin inhibit vaso-occlusion and inflammation in murine sickle cell disease: Role of heme oxygenase-1 induction. *PLoS ONE.* 2018;13(4):e0196455.
28. Thomsen JH, Etzerodt A, Svendsen P, Moestrup SK. The haptoglobin-CD163-heme oxygenase-1 pathway for hemoglobin scavenging. *Oxid. Med. Cell. Longev.* 2013;2013:523652.
29. Theurl I, Hilgendorf I, Nairz M, et al. On-demand erythrocyte disposal and iron recycling requires transient macrophages in the liver. *Nat. Med.* 2016;22(8):945–951.
30. White C, Yuan X, Schmidt PJ, et al. HRG1 Is Essential for Heme Transport from the Phagolysosome of Macrophages during Erythrophagocytosis. *Cell Metab.* 2013;17(2):261–270.
31. Korolnek T, Hamza I. Macrophages and iron trafficking at the birth and death of red cells. *Blood.* 2015;125(19):2893–7.
32. Soares MP, Hamza I. Macrophages and Iron Metabolism. *Immunity*. 2016;44(3):492–504.
33. Hubler MJ, Peterson KR, Hasty AH. Iron homeostasis: a new job for macrophages in adipose tissue? *Trends Endocrinol. Metab.* 2015;26(2):101–109.
34. Hubler MJ, Erikson KM, Kennedy AJ, Hasty AH. MFehi adipose tissue macrophages compensate for tissue iron perturbations in mice. *Am. J. Physiol. - Cell Physiol.* 2018;315(3):C319–C329.

35. Haschemi A, Kosma P, Gille L, et al. The sedoheptulose kinase CARKL directs macrophage polarization through control of glucose metabolism. *Cell Metab.* 2012;15(6):813–26.
36. O'Neill LAJ, Kishton RJ, Rathmell J. A guide to immunometabolism for immunologists. *Nat. Rev. Immunol.* 2016;16(9):553–565.
37. Wynn TA, Vannella KM. Macrophages in Tissue Repair, Regeneration, and Fibrosis. *Immunity*. 2016;44(3):450–462.
38. Serbulea V, Upchurch CM, Ahern KW, et al. Macrophages sensing oxidized DAMPs reprogram their metabolism to support redox homeostasis and inflammation through a TLR2-Syk-ceramide dependent mechanism. *Mol. Metab.* 2018;7:23–34.
39. Kenwood BM, Weaver JL, Bajwa A, et al. Identification of a novel mitochondrial uncoupler that does not depolarize the plasma membrane. *Mol. Metab.* 2014;3(2):114–123.
40. Bories GFP, Leitinger N. Macrophage metabolism in atherosclerosis. *FEBS Lett.* 2017;591(19):3042–3060.
41. Serbulea V, Upchurch CM, Schappe MS, et al. Macrophage phenotype and bioenergetics are controlled by oxidized phospholipids identified in lean and obese adipose tissue. *Proc. Natl. Acad. Sci. U. S. A.* 2018;201800544.
42. Kadl A, Meher AK, Sharma PR, et al. Identification of a novel macrophage phenotype that develops in response to atherogenic phospholipids via Nrf2. *Circ. Res.* 2010;107(6):737–46.
43. Nóbrega-Pereira S, Fernandez-Marcos PJ, Brioche T, et al. G6PD protects from oxidative damage and improves healthspan in mice. *Nat. Commun.* 2016;7:10894.
44. Tannahill GM, Curtis AM, Adamik J, et al. Succinate is an inflammatory signal that induces IL-1β through HIF-1α. *Nature*. 2013;496(7444):238–242.
45. Kim KS, Zhang D-L, Kovtunovych G, et al. Infused wild-type macrophages reside and self-renew in the liver to rescue the hemolysis and anemia of Hmox1-deficient mice. *Blood Adv.* 2018;2(20):2732–2743.

46. Pászty C, Brion CM, Manci E, et al. Transgenic Knockout Mice with Exclusively Human Sickle Hemoglobin and Sickle Cell Disease. *Science*. 1997;278(5339):876–878.
47. Ryan TM, Ciavatta DJ, Townes TM. Knockout-Transgenic Mouse Model of Sickle Cell Disease. *Science*. 1997;278(5339):873–876.
48. Wu L-C, Sun C-W, Ryan TM, et al. Correction of sickle cell disease by homologous recombination in embryonic stem cells. *Blood*. 2006;108(4):1183–8.
49. Vinchi F, Costa da Silva M, Ingoglia G, et al. Hemopexin therapy reverts heme-induced proinflammatory phenotypic switching of macrophages in a mouse model of sickle cell disease. *Blood*. 2016;127(4):473–486.
50. Riganti C, Gazzano E, Polimeni M, Aldieri E, Ghigo D. The pentose phosphate pathway: An antioxidant defense and a crossroad in tumor cell fate. *Free Radic. Biol. Med.* 2012;53(3):421–436.
51. Rochford R, Ohrt C, Baresel PC, et al. Humanized mouse model of glucose 6-phosphate dehydrogenase deficiency for in vivo assessment of hemolytic toxicity. *Proc. Natl. Acad. Sci. U. S. A.* 2013;110(43):17486–91.
52. Nairz M, Theurl I, Swirski FK, Weiss G. "Pumping iron"—how macrophages handle iron at the systemic, microenvironmental, and cellular levels. *Eur. J. Physiol.* 2017;469(3–4):397–418.
53. Tenhunen R, Marver HS, Schmid R. Microsomal heme oxygenase. Characterization of the enzyme. *J. Biol. Chem.* 1969;244(23):6388–6394.
54. Chi P-L, Lin C-C, Chen Y-W, Hsiao L-D, Yang C-M. CO Induces Nrf2-Dependent Heme Oxygenase-1 Transcription by Cooperating with Sp1 and c-Jun in Rat Brain Astrocytes. *Mol. Neurobiol.* 2015;52(1):277–292.
55. Motterlini R, Otterbein LE. The therapeutic potential of carbon monoxide. *Nat. Rev. Drug Discov.* 2010;9(9):728–743.
56. Foresti R, Bani-Hani MG, Motterlini R. Use of carbon monoxide as a therapeutic agent: promises and challenges. *Intensive Care Med.* 2008;34(4):649–658.

642 57. Ryter SW, Choi AMK. Targeting heme oxygenase-1 and carbon monoxide for therapeutic
643 modulation of inflammation. *Transl. Res. J. Lab. Clin. Med.* 2016;167(1):7–34.
644 58. Otterbein LE, Foresti R, Motterlini R. Heme Oxygenase-1 and Carbon Monoxide in the Heart: The
645 Balancing Act Between Danger Signaling and Pro-Survival. *Circ. Res.* 2016;118(12):1940–1959.
646 59. Yamamoto T, Takano N, Ishiwata K, et al. Reduced methylation of PFKFB3 in cancer cells shunts
647 glucose towards the pentose phosphate pathway. *Nat. Commun.* 2014;5:3480.
648 60. Chin BY, Jiang G, Wegiel B, et al. Hypoxia-inducible factor 1alpha stabilization by carbon monoxide
649 results in cytoprotective preconditioning. *Proc. Natl. Acad. Sci. U. S. A.* 2007;104(12):5109–5114.
650 61. Ganz T. Macrophages and systemic iron homeostasis. *J. Innate Immun.* 2012;4(5–6):446–53.
651 62. Deshmukh R, Trivedi V. Phagocytic Uptake of Oxidized Heme Polymer Is Highly Cytotoxic to
652 Macrophages. *PLoS ONE*. 2014;9(7):e103706.
653 63. Haldar M, Kohyama M, So AY-L, et al. Heme-Mediated SPI-C Induction Promotes Monocyte
654 Differentiation into Iron-Recycling Macrophages. *Cell*. 2014;156(6):1223–1234.
655 64. Kurotaki D, Uede T, Tamura T. Functions and development of red pulp macrophages. *Microbiol.*
656 *Immunol.* 2015;59(2):55–62.
657 65. da Guarda CC, Santiago RP, Fiuza LM, et al. Heme-mediated cell activation: the inflammatory
658 puzzle of sickle cell anemia. *Expert Rev. Hematol.* 2017;10(6):533–541.
659 66. Wang B, Cao W, Biswal S, Doré S. Carbon monoxide-activated Nrf2 pathway leads to protection
660 against permanent focal cerebral ischemia. *Stroke*. 2011;42(9):2605–10.
661 67. Yamamoto-Oka H, Mizuguchi S, Toda M, et al. Carbon monoxide releasing molecule-3 (CORM-3)
662 modulates progression of M1/M2 phenotypes in alveolar macrophages. *Free Radic. Biol. Med.*
663 2015;86:S20.
664 68. Correa-Costa M, Gallo D, Csizmadia E, et al. Carbon monoxide protects the kidney through the
665 central circadian clock and CD39. *Proc. Natl. Acad. Sci. U. S. A.* 2018;115(10):E2302–E2310.

69. Guo Y, Stein AB, Wu W-J, et al. Administration of a CO-releasing molecule at the time of reperfusion reduces infarct size in vivo. *Am. J. Physiol. - Heart Circ. Physiol.* 2004;286(5):H1649–53.
70. Fredenburgh LE, Perrella MA, Barragan-Bradford D, et al. A phase I trial of low-dose inhaled carbon monoxide in sepsis-induced ARDS. *JCI Insight*. 2018;3(23):.
71. Beckman JD, Belcher JD, Vineyard JV, et al. Inhaled carbon monoxide reduces leukocytosis in a murine model of sickle cell disease. *Am. J. Physiol.-Heart Circ. Physiol.* 2009;297(4):H1243–H1253.
72. Belcher JD, Young M, Chen C, et al. MP4CO, a pegylated hemoglobin saturated with carbon monoxide, is a modulator of HO-1, inflammation, and vaso-occlusion in transgenic sickle mice. *Blood*. 2013;122(15):2757–64.

Figure 1. Heme loading drives a bioenergetic shift in macrophages, reducing mitochondrial respiration and promoting glucose uptake. A. Bone marrow-derived macrophages (BMDMs) were treated with vehicle or heme (10 or 50μM) for 6 hours, and intracellular heme content normalized to protein (n=3). B. Mitochondrial Stress Test (MST) of BMDMs loaded with vehicle, 10μM, or 50μM heme for 6 hours (n = 3-4). The oxygen consumption rate (OCR) was measured initially (basal; blue), and after injection of 1μM oligomycin, 2μM BAM15 (maximal; green), and 10μM antimycin A and 1μM rotenone (gray; non-mitochondrial). Basal and maximal OCR were calculated by subtracting the mean OCR of the first three (basal) or post-BAM15 (maximal) measurements from the mean OCR of the post-AA/Rot measurements. Reserve capacity is the difference in maximal and basal respiratory capacity. C. Glucose uptake of BMDMs loaded with vehicle, heme (50μM) or lipopolysaccharide (LPS, 1μg/mL) for 6 hours, measured by accumulation of 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG) (n = 4). D. mRNA expression of *Glut1* in BMDMs loaded with vehicle or heme (50μM) for 6 hours measured by RT-qPCR (n = 4). E. Glycolytic stress test (GST) of BMDMs loaded with vehicle, 10μM, or 50μM heme for 6 hours (n = 4). The extracellular acidification rate (ECAR) was measured after injection of 20mM glucose, 1μM oligomycin, and 80mM 2-deoxyglucose (2-DG) to produce the basal (blue), stressed (green), and background (gray) ECAR, respectively. Basal and stressed ECAR were calculated by subtracting the mean ECAR of the post-glucose (basal) or post-Oligomycin (stressed) measurements from the mean ECAR of the post-2-DG measurements. F. Bioenergetics plot of heme-loaded macrophages, based on glycolytic capacity (stressed ECAR) and maximal respiratory capacity (Maximal OCR). G. Intracellular ATP levels of BMDMs treated with vehicle, 10μM, or 50μM heme as measured by luminescent signal of the Cell-Titer Glo ATP Assay, and represented as percent of initial vehicle control (n = 4). H. Viability of RAW264.7 macrophages treated with vehicle, 10μM, 50μM, or 100μM heme, determined by percentage of cells excluding trypan blue dye, and represented as percentage of initial vehicle control (n = 4). Data are represented as mean ± SEM. Statistical significance between 2 groups was determined by Welch's unpaired t-test. Significance between more than 2 groups was determined by One-Way ANOVA, with post-hoc

716 Tukey or Dunnett's Multiple Comparison Test to determine significance between groups. * represents
717 $p<0.05$, ** represents $p<0.01$, and *** represents $p<0.001$.
718
719 **Figure 2. Unbiased metabolomics analysis of heme loaded macrophages reveals enrichment for**
720 **metabolites associated with glycolysis and the pentose phosphate pathway.** A. Volcano plot
721 representing the changes in bone marrow-derived macrophage (BMDM) metabolism after 6 hours of 10μM
722 heme loading, as measured by unbiased mass-spectrometry based metabolomics. Log2 of the fold change
723 of individual metabolites are plotted on the x-axis, and -Log2 of the p-value of a Welch's t-test between
724 control and heme-loaded BMDMs is plotted on the y-axis. (n = 5). B. Changes in heme metabolite
725 abundance in heme-loaded BMDMs. Metabolites in red text are increased in heme loaded BMDMs relative
726 to vehicle control, with corresponding plots for the individual metabolites involved in heme catabolism (n
727 = 5). C. Plots representing individual metabolites involved in the glycolysis pathway (n = 5). D. Plots
728 representing individual metabolites involved in the pentose phosphate pathway (n = 5). E. Pathway analysis
729 summary of glycolysis and pentose phosphate pathways in heme-loaded BMDMs. Metabolites in red text
730 are increased in heme-loaded BMDMs relative to vehicle control. Data are mean ± SD of relative abundance
731 measurements for control and heme-loaded BMDMs (n=5). Statistical significance between 2 groups was
732 determined by Welch's unpaired t-test. * represents $p<0.05$, ** represents $p<0.01$, and *** represents
733 $p<0.001$. See also Supplementary Figure 1, Related to Figure 2.
734
735 **Figure 3. Heme loading induces changes in metabolic gene expression to promote persistent pentose**
736 **phosphate pathway flux and macrophage redox homeostasis.** A. Fold change in individual genes
737 involved in the glycolytic, pentose phosphate, or heme metabolism pathways, as measured by RT-qPCR of
738 BMDMs treated with 1, 5, or 10μM heme for 6 or 24 hours (n = 3). Data represented in a heat map showing
739 the fold change compared to vehicle-treated macrophages. Primer sequences can be found in Table S1. B.
740 Volcano plots representing gene upregulation in BMDMs treated with 10μM heme for 6 or 24 hours (n=3),
741 with horizontal cutoff for $p<0.05$. PPP and related enzymes are highlighted in purple. C. Pathway analysis

742 summary of 10μM heme-driven gene regulation in BMDMs. mRNA for each gene was measured after 6
743 hours (left box) and 24 hours (right box) of heme stimulation. Orange-shaded boxes indicate upregulation
744 while blue-shaded boxes indicate downregulation of the respective gene versus the corresponding vehicle
745 control. D. mRNA expression of *G6pd* and *Pgd* in BMDMs treated with 1-10μM heme for 6 or 24 hours
746 (n = 4). B. G6PD Activity, measured as the rate of NADPH synthesis, in BMDMs treated with vehicle or
747 10μM heme for 6 or 24 hours (n = 4). F. Intracellular concentrations of reduced NADPH of BMDMs treated
748 with vehicle, 100μM dehydroepiandrosterone (DHEA; an inhibitor of G6PD), and/or 10μM heme (n = 4).
749 G. Levels of oxidized glutathione (GSSG) in macrophages treated with vehicle, 100μM DHEA and/or
750 10μM heme (n = 4). H. Ratio of reduced glutathione (GSH) to GSSG in BMDMs treated with vehicle,
751 100μM DHEA and/or 10μM heme (n = 4). Data are represented as mean ± SEM. Statistical significance
752 between 2 groups was determined by Welch's unpaired t-test. Significance between more than 2 groups
753 was determined by One-Way ANOVA, with post-hoc Tukey's or Dunnett's Multiple Comparison Tests, as
754 appropriate, to determine differences between specific groups. * represents $p<0.05$, ** represents $p<0.01$,
755 and *** represents $p<0.001$. See also Supplementary Figure 2 and Supplementary Tables 1 and 2, Related
756 to Figure 3.
757
758 **Figure 4. Glucose-6-phosphate dehydrogenase activity is required for heme detoxification in**
759 **macrophages.** A. BMDMs were treated with 20μM heme and/or 100μM DHEA, and intracellular heme
760 concentration measured at 1, 3, and 6 hours (n = 4). B. BMDMs were treated with 20μM heme for 4 hours
761 to create heme-loaded macrophages. Subsequently, heme loaded BMDMs were washed and treated with
762 vehicle or 100μM DHEA, and intracellular heme was measured at 5, 6, 8, 10, and 16 hours (n=4). C. The
763 difference in intracellular heme at 4 and 16 hours (representing 12 hours of degradation) was used to
764 calculate the heme degradation rate in heme-loaded macrophages +/- the addition of DHEA (n = 4). D.
765 Schematic representing the requirement of G6PD activity for macrophage heme degradation. Data are
766 represented as mean ± SEM. Statistical significance between 2 groups was determined by Welch's unpaired

t-test. Significance between more than 2 groups was determined by One-Way ANOVA, with post-hoc Tukey's tests to determine differences between specific groups. * represents $p<0.05$, ** represents $p<0.01$, and *** represents $p<0.001$. See also Supplementary Figure 3, Related to Figure 4.

Figure 5. Carbon monoxide generated by HO-1-dependent heme breakdown upregulates the pentose phosphate pathway. A. Schematic showing the release of CO from CORM3 upon reaction with water, and the generation of inactivated CORM3 (iCORM3). B. mRNA expression of *Glut1*, *G6pd*, and *Pgd* in BMDMs treated with vehicle, 35-70μM CORM3, or 35-70μM iCORM3 for 6 hours. (n = 4). Corresponding, representative western blot analysis of G6PD and PGD protein in BMDMs treated with vehicle, 100μM iCORM3 (iCO), or 50-100μM CORM3 (CO) for 12 and 24 hours. C. G6PD activity, measured as the rate of NAPDH synthesis, in BMDMs treated with vehicle, 100μM CORM3, or 100μM iCORM3 for 6 or 24 hours (n = 3-4). D. Intracellular NADPH levels measured from BMDMs treated with vehicle, 50-100μM CORM3, or 50-100μM iCORM3 for 6hours (n=4). E. mRNA expression of 'M1' macrophage markers in BMDMs treated with vehicle, iCORM3, or CORM3 (50μM) for 6 hours (N=4). F. mRNA expression of 'M2' macrophage markers in BMDMs treated with vehicle, iCORM3, or CORM3 (50μM) for 6 hours (N=4). G. mRNA expression of 'Mox' macrophage markers in BMDMs treated with vehicle, iCORM3, or CORM3 (50μM) for 6 hours (N=4). H. Schematic representing feed-forward regulation of PPP by heme breakdown, mediated by CO. Data are represented as mean ± SEM. Statistical significance between 2 groups was determined by Welch's unpaired t-test. Significance between more than 2 groups was determined by One-Way ANOVA, with post-hoc Tukey's or Dunnett's multiple comparisons tests, as appropriate, to determine differences between specific groups. * represents $p<0.05$, ** represents $p<0.01$, and *** represents $p<0.001$. See also Supplementary Figure 4, related to Figure 5.

Figure 6. Carbon monoxide mimics the metabolic phenotype of heme-loaded macrophages. A. Mitochondrial stress test (MST) of BMDMs treated with vehicle, 100μM iCORM3, or 100μM CORM3 for 6 hours ($n \geq 3$). The oxygen consumption rate (OCR) was measured initially (basal; blue), and after injection

of 1μM oligomycin, 2μM BAM15 (maximal; green), and 10μM antimycin A and 1μM rotenone (gray; non-mitochondrial). Basal and maximal OCR were calculated by subtracting the mean OCR of the first three (basal) or post-BAM15 (maximal) measurements from the mean OCR of the post-AA/Rot measurements. Reserve Capacity is the difference in maximal and basal respiratory capacity. B. Glycolytic stress test (GST) of BMDMs treated with vehicle, 100μM iCORM3, or 100μM CORM3 for 6 hours ($n \geq 3$). The extracellular acidification rate (ECAR) was measured after injection of 20mM glucose, 1μM oligomycin, and 80mM 2-deoxyglucose (2-DG) to produce the basal (blue), stressed (green), and background (gray) ECAR, respectively. Basal and stressed ECAR were calculated by subtracting the mean ECAR of the post-glucose (basal) or post-Oligomycin (stressed) measurements from the mean ECAR of the post-2-DG measurements. C. Bioenergetics plot relates the cells' respiratory capacity (based on maximal OCR, y-axis) to the glycolytic capacity (based on stressed ECAR, x-axis), in BMDMs treated with CORM3 or iCORM3 for 6 hours ($n \geq 3$). D. as measured by luminescent signal of the Cell-Titer Glo ATP Assay, and represented as percent of initial vehicle control ($n = 5$). Data are represented as mean ± SEM. Statistical significance between 2 groups was determined by Welch's unpaired t-test. Significance between more than 2 groups was determined by One-Way ANOVA, with post-hoc Tukey's or Dunnett's multiple comparisons tests, as appropriate, to determine differences between specific groups. * represents $p<0.05$, ** represents $p<0.01$, and *** represents $p<0.001$. See also Supplementary Figure 5, related to Figure 6.

Figure 7. Macrophages undergo metabolic adaptation in an *in vivo* model of hemolytic SCD. A. Blood was collected from transgenic B6/129 knock-in mice (Townes SCD model) expressing human hemoglobin α and homozygous for either the β^A or β^S sickle hemoglobin, and serum heme concentrations measured ($n = 5$ per group). B. mRNA expression of *Hmox1*, *G6pd*, and *Pgd* in spleens of β^A or β^S mice ($n = 5$ per group). C. mRNA expression of *Hmox1*, *G6pd*, and *Pgd* in livers of β^A or β^S mice ($n = 5$). D. G6PD Activity, measured as the rate of NADPH synthesis, in supernatants of whole liver lysates of β^A or β^S mice ($n = 5$ per group). E. Correlation of serum heme concentration with liver G6PD activity in β^A or β^S mice ($n = 5$

per group). F. Representative gating strategy for flow-assisted cell sorting (FACS) of macrophage-like cells from spleens of β^A or β^S mice. G. Abundance of macrophages, as percentage of live, $CD45^+$ cells from β^A or β^S mice (n = 4). H. mRNA expression of *G6pd* and *Pgd* in FACS-sorted splenic macrophages from β^A or β^S mice. I. Schematic describing CORM3 treatment regimen and peritoneal cell isolation. J. mRNA expression of G6pd and *Pgd* in isolated peritoneal cells from mice treated with saline or CORM3 for 7 days (n = 5). Data are represented as mean ± SEM. Statistical significance between 2 groups was determined by Welch's unpaired t-test. Significance between more than 2 groups was determined by One-Way ANOVA, with post-hoc Tukey's or Dunnett's multiple comparisons tests, as appropriate, to determine differences between specific groups. Correlation analysis is based on determination of Pearson coefficient, and significant correlations are presented as R^2 values. * represents $p<0.05$, ** represents $p<0.01$, and *** represents $p<0.005$.